AN INTRACELLULAR POLYSACCHARIDE THAT SERVES AS A CARBON AND ENERGY SOURCE FOR SPORULATION IN *BACILLUS CEREUS* STRAIN T

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by

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An abstract of a Thesis by
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The Problem. *Bacillus cereus* strain T accumulates an intracellular polysaccharide during late logarithmic and early stationary phases of the growth cycle. The polysaccharide is subsequently degraded during the sporulation phase of the growth cycle. Initial research suggested that the polysaccharide was used as a carbon and energy storage compound for the completion of sporulation. Subsequent work involved using polysaccharide-minus mutants of the strain to support this hypothesis. This paper details the use of a mutant deficient in its ability to degrade the polysaccharide to verify the original hypothesis.

Procedure. Spores of *Baccilus cereus* strain T were treated with the chemical mutagen ethyl methane sulfonate and screened for the ability to accumulate and not degrade the intracellular polysaccharide. A suitable mutant (CH-5) was isolated and compared to the wild-type in terms of growth characteristics, polysaccharide and poly-β-hydroxy butyrate (PHB) accumulation and degradation, spore heat resistance, and the ability to sporulate endotrophically.

Findings. The mutant strain (CH-5) demonstrated growth characteristics almost identical to those of the wild-type strain (T). Polysaccharide and PHB accumulation were very similar in both strains under similar conditions but CH-5 was unable to degrade the polysaccharide during spore formation. Mutant spores, produced in the absence of intracellular PHB, were much less heat resistant than wild-type spores produced under the same conditions. The mutant strain was also unable to sporulate endotrophically when grown under conditions preventing PHB accumulation.

Conclusions. The intracellular polysaccharide of *Bacillus cereus* strain T functions as a source of carbon and energy for sporulation, especially under conditions preventing PHB accumulation. The polysaccharide also appears to contribute to increased heat resistance of spores, possibly by an osmotic mechanism.

Recommendations. It might be of interest to obtain mutants blocked at various stages of sporulation to see what effect this has on polysaccharide accumulation and degradation.

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INTRODUCTION

The value of carbon and energy storage compounds. Carbon and energy storage compounds are of widespread occurrence in bacteria. Wilkinson (1959) suggested at least five uses of carbon and energy storage compounds in the bacterial cell: (1) for cell growth in the absence of an exogenous source of carbon and energy; (2) for special phases of the cell division cycle; (3) for the maintenance of cell integrity and viability; (4) for energy required in adaptation to a different growth medium; and (5) for special mechanisms of survival, such as, motility or sporogenesis. Wilkinson (1959) also suggested that a compound must fulfill three criteria in order to be considered an internal source of carbon and energy: (1) the compound must accumulate when the exogenous nutrient source is in greater supply than is needed for cell growth; (2) it must be used as the exogenous source falls below the level necessary for cell growth or maintenance; and (3) it must have a role in the ability of the organism to survive.

Two types of carbon and energy storage compounds. Two types of substances, occurring in bacteria, have been shown to serve as carbon and energy storage compounds. They are polysaccharides and lipids (Wilkinson, 1959). Polysaccharides can be of the extracellular (capsular) or intracellular type, while lipds can be in the form of

triglycerides or poly-β-hydroxy butyrate (PHB). This report will deal with polysaccharides and PHB.

Both intracellular and extracellular polysaccharides are formed to the greatest extent in media having an excess of carbon and energy source and a deficiency of some nutrient not required for polysaccharide synthesis (Wilkinson, 1959).

Extracellular polysaccharides do not generally appear to serve as reserve sources of carbon and energy since most organisms are unable to degrade these compounds once they are formed. This is most likely due to the irreversibility of the synthetic reactions, or to the spatial separation of the polysaccharides from the protoplasmic enzymes.

Holme and Palmstierna (1956 a,b,c) used Escherichia coli B, which accumulates intracellular glycogen, as a model for studying the use of an intracellular polysaccharide as a reserve source of carbon and energy. Cells were grown in media that was deficient in either the source of carbon and energy, nitrogen (Holme and Palmstierna, 1956a), phosphorous, or sulfur (Holme and Palmstierna, 1956b). The stationary phase cultures in nitrogen deficient media had the highest levels of glycogen. After a period of starvation, a quantity of the limiting nutrient was added to the medium. This resulted in a decrease in the glycogen content and a corresponding increase in cell mass.

Radioactive tracers were also used to show the use of glycogen-carbon for synthetic purposes (Holme and Palmstierna, 1956c). Cells were grown to stationary phase in nitrogen deficient media and then incubated for a period with ^{14}C-lactate or ^{14}C-glucose. Cells were then cooled, centrifuged, and washed. A significant amount of labelled glycogen had been produced compared to a small amount of labelled protein. Transfer of the cells to a carbon-free, nitrogen containing media resulted in partial utilization of the glycogen and a corresponding net increase in the synthesis of labelled protein.

Zevenhuizen (1966) and Mulder and Zevenhuizen (1967) working with organisms of the genus Arthrobacter used experiments similar to those of Holme and Palmstierna (1956 a,b) to demonstrate that the greatest accumulation of polysaccharide was produced in cells grown in a medium rich in glucose and deficient in nitrogen, phosphorous, or sulfur.

Poly-β-hydroxy butyrate (PHB) has been shown to be a compound of widespread occurrence in bacteria, especially in Bacillus species (Wilkinson, 1959). Under ideal conditions, PHB may make up over 50 percent of the cell dry weight. PHB granules are larger and more numerous in cells grown in media containing an excess of carbon and energy source and a limiting amount of nitrogen, phosphorous, sulfur, or potassium (Wilkinson, 1959).

The importance of carbon and energy storage compounds in spore-forming bacteria. Bacterial spore formation is associated with the exhaustion of some exogenous nutrient from the culture medium. Under these conditions, a cell must use its own reserves to provide sufficient nutrients to complete the sporulation process.

Day and Costilow (1964a) did not observe any cytoplasmic starch granules and did not detect any PHB in vegetative cells of Clostridium botulinum type A. Further investigation demonstrated that an exogenous source of energy was required for forespore formation in C. botulinum type A (Day and Costilow, 1964b). Similar studies showed that C. thermosacchrolyticum also requires an exogenous source of carbon and energy to complete the sporulation process (Hsu and Ordal, 1969).

Bacillus licheniformis and B. subtilis do not store PHB nor any detectable amount of polysaccharide and they do not sporulate endotrophically (Slepecky and Law, 1961a; Williamson and Wilkinson, 1958).

Vegetative cells of C. botulinum type E (Minnesota) have been shown to accumulate a starch-like glucan during growth in glucose-trypticase medium (Strasdine, 1968). This particular strain undergoes a rapid, almost synchronous, formation of spores at the end of logarithmic growth and the intracellular concentration of polysaccharide decreases

during this phase of the cell cycle. This lead the author to suggest that the intracellular polysaccharide served as a source of carbon and energy during sporulation of the strain (Strasdine, 1968).

Clostridium pasteurianum accumulates large amounts of granulose, an amylopectin-like α-polyglucosan, at the beginning of stationary phase when grown in a glucose rich medium (Mackey and Morris, 1974), and the polyglucan is degraded when the organisms are transferred to a medium devoid of any exogenous source of carbon and energy (Robson et al., 1974). In this organism, degradation of granulose does not occur in the presence of exogenous glucose. The granulose of C. pasteurianum can serve as an endogenous reserve material for sporulation under appropriate conditions (Dawes and Senior, 1973).

Further studies by Morris and Robson (1973), using granulose-negative mutants of C. pasteurianum demonstrated that granulose synthesis itself served a useful purpose. These mutants sporulated poorly in glucose-rich minimal medium but they sporulated well when the rate of supply of glucose was so low as to sustain sporulation of the parent, wild-type organism without concurrent production of granulose. No other endogenous reserve of glucose was observed in the organism and these workers suggested that granulose synthesis in C. pasteurianum served as an

"overflow" mechanism to moderate increases in the intracellular levels of potentially harmful metabolites (including suppressor(s) of sporulation) that could arise during stationary phase in a glucose rich medium.

Although several intestigators have established that PHB can serve as a source of carbon and energy (Dawes and Ribbons, 1962; Komineck, 1964; Nakata, 1963, 1966; Slepecky and Law, 1961a), they also conclude that PHB production is not mandatory for sporulation in Bacillus cereus and B. megaterium. Komineck (1964) found that very little PHB accumulated in cells of B. cereus grown in G-medium buffered in pH 7.4. Under these conditions, the efficiency of sporulation is equivalent to that of cells grown in weakly buffered G-medium in which cells accumulated large amounts of PHB. These two types of cells also had the same efficiency of sporulation when placed under conditions of endotrophic sporulation. This suggests that PHB is not mandatory for sporulation.

Aubert (1951) has shown that B. megaterium produces an intracellular polysaccharide identified as glycogen by Barry et al. (1953). Tinelli (1955) found that B. megaterium produces both a polysaccharide and PHB making up 20% and 28%, respectively, of the cell dry weight at the beginning of stationary phase. These values decreased to 12% and 18%, respectively, of the cell dry weight at the

time of appearance of refractile spores.

Slock and Stahly (1974) showed that B. cereus also produces an intracellular polysaccharide in addition to PHB. Further work has demonstrated a pattern of accumulation and degradation similar to that of B. megaterium (Berka, 1979; Schmid, 1980; Slock and Stahly, 1974). The levels of polysaccharide and PHB peak during early stationary phase and decline as sporulation proceeds. This phenomenon of accumulation and degradation occurred when growth was limited by glucose or a component of yeast extract. Their data suggested that the polysaccharide could function as an endogenous source of carbon and energy for sporulation in B. cereus (Berka, 1979; Schmid, 1980; Slock and Stahly, 1974). Their work also pointed out that, unlike PHB, polysaccharide accumulation was the same in strongly and weakly buffered media and they postulated that the polysaccharide was the major carbon and energy storage compound for cells grown under conditions preventing significant accumulation of PHB. This hypothesis was supported by Berka (1979) using a polysaccharide-minus mutant of B. cereus. Under conditions of endotrophic sporulation, this mutant sporulated normally when grown in weakly buffered medium which allowed PHB accumulation but did not sporulate endotrophically when grown in a medium buffered at pH 7.4. The polysaccharide from B. cereus was purified (Berka, 1979;

Slock and Stahly, 1974) and found to consist of glucose in α-1,4 linked glucopyranose units with occasional α-1,4,6 linked branches. The degree of branching was found, by iodine absorption spectroscopy, to be between that of glycogen and amylopectin (Berka, 1979; Slock and Stahly, 1974).

It is well known that the heat resistance of bacterial spores is influenced markedly by the composition of the heating medium. High molecular weight substances such as gelatin, starch, gum arabic, and alginic acid have been shown to exert a heat protective effect on spores of B. mesentericus and B. brevis (Kawai and Tanaka, 1952). But Amaha and Sakaguchi (1954) observed no additional heat resistance in spores of Clostridium sporogenes with high molecular weight compounds such as casein, pectin, dextrin, and sugars (P.A. 3679).

The degradation of the intracellular polysaccharide prior to spore maturation in some species may be a significant observation since it has been established that high sugar concentrations increase the heat resistance of bacteria (Stumbo, 1965). Flowers and Adams (1976) observed that a liquid repair medium containing 10% sucrose increased the number of survivors for ultrahigh temperature-injured spores of Clostridium perfringens. Busta, Baillie, and Murrell (1976) showed that a low concentration of sucrose

(0.6M) in an enumeration medium led to a higher number of survivors of heated B. cereus forespores. Schmid (1980) and Berka (1979) found that a strain of B. cereus which produced an intracellular polysaccharide, appeared to be more heat resistant than polysaccharide-minus mutants of the strain. However, Berka (1979) also showed that cells grown under conditions allowing polysaccharide and PHB accumulation and then allowed to sporulate endotrophically were more heat resistant than cells allowed to sporulate in the growth medium. Berka (1979) postulated that the breakdown of the intracellular polysaccharide to glucose in the mother cell during sporulation could raise the osmotic pressure enough to significantly aid in dehydration of the forespore, resulting in increased thermostability.

Schmid (1980) showed that a polysaccharide-minus mutant of B. cereus, grown under conditions allowing PHB accumulation, were more heat resistant with a low level of glucose in the endotrophic sporulation medium than without glucose present.

This paper will concern itself with verifying that the intracellular polysaccharide of Bacillus cereus indeed functions as a carbon and energy storage compound for use during sporulation. The role of the polysaccharide in heat resistance of the spores will also be addressed.

MATERIALS AND METHODS

Organism. Bacillus cereus strain T (B.c.T) is the wild-type organism used throughout this study. A mutant, deficient in the breakdown of the intracellular polysaccharide, and presumably normal in all other aspects of metabolism, was obtained from the wild-type using the method of Ito and Spizizen (1971). This method involved suspending approximately $1x10^8$ spores per ml in 0.1M potassium phosphate buffer (pH 7.0), heat shocking the spores for 15 minutes at 70°C, cooling the suspension, and adding the chemical mutagen ethyl methane sulfonate (EMS) to a final concentration of 0.45M. The suspension was then shaken at 30°C for four hours before being centrifuged, washed twice in potassium phosphate buffer, and resuspended in distilled water. Survival of the spores was approximately 10%. The treated spores were frozen until needed.

Mutagen-treated spore stock was thawed and grown to mid-exponential phase (A_{595}=0.4) using the active culture technique (below). Cells were then harvested by centrifugation, resuspended in distilled water, serially diluted, and plated on a glucose-deficient agar medium described below. Plates were incubated at 30°C for 48 hours and then inverted over I_2 crystals. Wild-type colonies stained brick-red around the edges and were light in the center

indicating polysaccharide degradation. Mutant colonies selected remained brick-red throughout, even after five days incubation.

A total of five colonies were selected and screened for their ability to grow and sporulate normally. Mutant CH-5 exhibited characteristics most like that of the wild-type and was used for the remainder of these studies.

A modified agar medium was developed for use in identifying desirable mutants. A 10% transfer of an exponentially growing culture of B.c.T was made to a flask of G-medium (below) and incubated at 30°C and 200 rpm on a rotary shaker for approximately four hours in order to deplete the glucose supply. Cells were harvested at 8000 rpm for 15 minutes at 4°C and the supernatant aseptically filtered through a 0.22 micrometer pore size Millipore filter (Nalgene). A concentrated solution of Bacto Agar (Difco) was autoclaved and aseptically added to the filtrate at 55°C to give a final agar concentration of 1.5 g/L. Plates were poured, allowed to solidify, and refrigerated until needed.

Growth and sporulation media. N-medium was developed by Slock (1970) for the growth of Bacillus cereus T (B.c.T). Polysaccharide production by B.c.T in N-medium is increased due to the excess supply of glucose and the limiting supply of nitrogen.

Stock solutions of 10% glucose (Difco), 10% yeast extract (Difco), 5% K_2HPO_4 (Fisher Chemical Co.), 0.8% $CaCl.2H_2O$ (Fisher Chemical Co.) were prepared and autoclaved. A stock mineral solution was prepared by combining 0.1% $FeSO_4.7H_2O$ (J.T. Baker Chemical Co.), 0.1% $CuSO_4.5H_2O$ (Fisher Chemical Co.), 1.0% $MnSO_4.H_2O$ (Fisher Chemical Co.), 0.1% $ZnSO_4.7H_2O$ (Fisher Chemical Co.), and 4.0% $MgSO_4$ (Fisher Chemical Co.) in distilled water with an equal volume of 40.0% $(NH_4)_2SO_4$ (Mallinckrodt Chemical Works).

The medium was prepared by adding mineral solution to a predetermined volume of distilled water to give a final concentration of 1.0% (v/v). This solution was autoclaved and allowed to cool to room temperature. Prior to inoculation, stock solutions of glucose, yeast extract, potassium phosphate, and calcium chloride were aseptically added to give final concentrations of 2.0%, 1.0%, 1.0%, and 1.0%, respectively. The final pH of the medium was 7.2-7.4. The composition of the medium is shown below.

Phosphate buffered G-medium (PBG), developed by Kominek (1964) from G-medium (Greenberg, 1954), was formulated to prevent the accumulation of poly-β-hydroxy butyrate by B. cereus strain T. The same stock solutions described for N-medium were used, except 1.0M potassium phosphate buffer was substituted for the 5% K_2HPO_4. The final concentrations of this medium were: 1.0% glucose, 1.0% calcium

chloride, 2.0% yeast extract, 1.0% mineral salts solution, and 0.1M potassium phosphate buffer. The final pH was 7.4.

Compound	Percent (w/v)
$FeSO_4.7H_2O$	0.00005
$ZnSO_4.7H_2O$	0.0005
$CuSO_4.5H_2O$	0.0005
$MnSO_4.H_2O$	0.005
$CaCl_2.2H_2O$	0.008
$MgSO_4$	0.02
K_2HPO_4	0.05
Yeast Extract	0.10
$(NH_4)_2SO_4$	0.20
Glucose	0.20

Growth conditions. The "active culture technique" (Collier, 1957), as modified by Halvorson (1957) for growth and sporulation of B. cereus strain T, was used throughout this study. This technique employs several transfers of exponential phase cells to fresh medium in order to select for those cells that are growing most rapidly. This results in a more synchronous culture with regards to cell morphology and physiology.

Frozen spore stocks (approximately 10^9 spores per ml) of the organisms were maintained and thawed as needed. The starter culture was prepared by inoculating 0.1 ml of a 10^{-7} dilution of spore stock into the desired growth

medium. The culture was incubated at 30°C and 200 rpm on a rotary shaker (New Brunswick Scientific Co.) until the absorbance at 595 nm was 0.3-0.4 (approximately 9 hours). A volume of starter culture was transferred to fresh culture medium to give a final concentration of 10%. This second culture was incubated as before until the absorbance reached 0.3-0.4. A final 10% transfer was then made to the experimental flasks of growth medium.

Examination of cultures. Culture samples were routinely examined for cell morphology and cell counts using a Petroff-Hausser counting chamber and a phase contrast microscope (American Optical Co.).

Measurement of pH and turbidity. The pH of the cultures was measured using a Corning Model 10 pH meter. Culture turbidity was measured using a Bausch and Lomb Spectronic 20 spectrophotometer.

Harvesting of organisms. Duplicate 100 ml samples were harvested by centrifugation in a Sorvall RC3-B (DuPont) refrigerated centrifuge at 4°C using a GSA rotor. The samples were washed twice with sterile distilled water and frozen.

Dry weight determination. Cell dry weights were determined by harvesting 100 ml of culture, resuspending the cells to 100 ml in distilled water, and placing 20 ml in pre-weighed aluminum foil pans. Samples were dried to

constant weight in a 100° oven. The difference between tare weight and dry weight was multiplied by 5 to give cell dry weight (mg) per 100 ml culture.

Enzymatic glucose determination. Frozen cell samples were suspended in 5 ml of 2.0 N H_2SO_4 and hydrolyzed in a boiling water bath for three hours. The samples were cooled, centrifuged to remove insolubles and washed with 5 ml distilled water. The supernatants were combined and the pH adjusted to 7.0 with 2.0M K_2HPO_4. The final volume was recorded and glucose concentration determined enzymatically. Glucose concentration in samples was determined using Sigma No. 510-A Colorimetric enzymatic glucose assay kit (Sigma Chemical Co.). A series of test tubes was prepared which included one for each standard, one for each unknown and one for a reagent blank. A volume of 0.5 ml of a standard, hydrolysate, or distilled water was pipetted into the respective tubes. Each sample tube received 5.0 ml of enzyme-color reagent and all tubes were incubated at 37°C for 30 minutes. Absorbance was read at 450 nm, against a reagent blank, in a Beckman ACTA UV/visible spectrophotometer. Glucose concentration in the hydrolysed samples was determined using the following formula:

$$\frac{A_{sample}}{A_{standard}} \times \text{final volume pH 7.0 hydrolysate (ml)} = \frac{\text{mg glucose}}{\text{100 ml culture}}$$

Assay of cell PHB content. Cellular PHB content was determined spectrophotometrically after its isolation and conversion to crotonic acid in concentrated sulfuric acid (Slepecky and Law, 1961b). Five ml aliquots were taken from washed 100 ml frozen cell samples and centrifuged. The pellets were suspended in 5.0 ml of sodium hypochlorite (Clorox) and incubated for one hour in a 37° water bath. The samples were centrifuged and washed with 5.0 ml each of water, acetone, and ethanol, all near their boiling points. The sample pellets were fully suspended in each wash using a Vortex mixer and centrifuged. All supernatants were discarded. PHB was extracted using three 3.0 ml portions of boiling chloroform. The pellets were suspended in each portion and placed in a boiling water bath for three minutes before centrifugation. All supernatants were combined and passed through a glass microfiber filter (Whatman GF/A, Fisher Scientific Co.) which had been prewashed with hot chloroform. A 3.0 ml portion of hot chloroform was used to clear the filter between samples. The chloroform was evaporated from the samples in a boiling water bath and 20 ml of concentrated H_2SO_4 was added to each sample tube. The tubes were capped with marbles, allowed to reflux at 100°C for 10 minutes, and then cooled to room temperature. Absorbance of the samples was read at 235 nm, against a H_2SO_4 blank, and the concentration calculated using the molar extinction coefficient of crotonic acid (1.56×10^4).

An absorbance scan was used to verify a single absorbance peak at 235 nm representing crotonic acid.

Determination of spore heat resistance. Heat resistance of spores grown in N and PBG media was determined by placing suspensions of approximately 10^6 spores per ml in an 80°C water bath. Samples were aseptically removed every 10 minutes and cooled immediately in an ice bath. Serial dilutions were made in sterile water blanks and the survivors enumerated by the pour-plate method. Plates were counted after 20 hours incubation at 30°C.

The logarithm of the surviving fraction of the population [$\log S_t/S_o$=log (colony forming units per ml at t minutes/colony forming units per ml at 0 minutes)] was plotted against time for exposure to give a survivor curve. The heat resistance was expressed as a decimal reduction time (D-value). The D-value was the time required at a given temperature to reduce the number of viable organisms by 90% i.e., the time required for the log survivor curve to transverse one log cycle, and was mathematically equivalent to the inverse of the slope of the log survivor curve (Roberts and Hitchins, 1969).

Endotrophic sporulation studies. Using the active culture technique, cells were grown one hour into stationary phase (approximately 5 hours) after the final transfer. Samples were harvested aseptically and washed twice with sterile distilled water. The cells were resuspended to the

original sample volume in sterile 0.008% $CaCl_2.2H_2O$ solution in sterile 250 ml Erlenmeyer flasks. The cell suspensions were incubated at 30°C for 12 hours on a rotary shaker. The efficiency of endotrophic sporulation was determined by direct counts of the cell suspensions in a Petroff-Hausser bacteria counting chamber before and after the incubation period, and was defined as the number of spores formed divided by the number of cells originally present.

RESULTS AND DISCUSSION

Organisms. Bacillus cereus strain T (B.c.T) was the wild-type organism used for comparison in this study. A mutant, CH-5, deficient in its ability to catabolize the intracellular polysaccharide, was obtained by ethyl methane sulfonate (EMS) mutagenesis of the wild-type organism, as explained in Materials and Methods, and was used as the experimental organism throughout this study.

Growth in N- and PBG-media. Detailed accounts of changes in cell morphology and metabolism taking place in B.c.T during the transition from vegetative to sporulating cells have been presented by Halvorson (1957), Nakata and Halvorson (1960), Hashimoto et al. (1960), Hanson (1962), Hanson et al. (1963a,b), Srinivasan and Halvorson (1963), and Kominek (1964). Only a brief description of some of these changes will be given as a basis for some observations and discussion.

The two media used for the growth and sporulation of B.c.T and CH-5 were N-medium (Slock, 1970) and PBG-medium (Kominek, 1964). Growth in N-medium was presumably limited by nitrogen and not the carbon and energy source (glucose). This led to enhanced production of the intracellular polysaccharide and poly-β-hydroxy butyrate (PHB) storage compounds. In PBG-medium, polysaccharide production decreased due to the limiting concentration of glucose, acting as the primary source of carbon and energy, and PHB accumulation was reduced due to the strong pH 7.4 buffer.

Holme and Cedergren (1961) observed diffuse, transparent areas in the cytoplasm of E. coli and identified them as areas of glycogen accumulation. Similar "spots" observed in B. cereus (Slock, 1970) were presumed to be areas of polysaccharide accumulation although no studies have been performed to verify this.

Table 1 shows the major morphological characteristic of B.c.T and CH-5 observed by phase constrast microscopy at various stages of development in N- and PBG-media. The two strains showed essentially identical characteristics except for a more granular cytoplasm of CH-5, especially after 10 hours, due to the continued presence of relatively large amounts of intracellular polysaccharide. As expected, the cells growing in PBG-medium did not contain noticeable PHB granules.

Figures 1A, B, and C illustrate the pH of the medium,

Table 1. Microscopic morphology of Bacillus cereus T or CH-5 cells as a function of incubation time in N- and PBG-media.

Hour	N-Medium	PBG-Medium
0-3	Chains; short rods; homogenous cytoplasm	Chains; short rods; homogenous cytoplasm
4	Short chains; cytoplasm becoming granular	Short chains; homogenous cytoplasm
5	PHB granules evident	Granular cytoplasm; no large granules
6	PHB granules	No change
7	PHB granules; forespores forming	No change
8	Forespores becoming refractile; PHB granules; granular cytoplasm	Forespores forming; granular cytoplasm
10	Some refractile spores; fewer granules	Forespores becoming refractile
12	Majority refractile spores; few granules	Refractile spores; granular cytoplasm (CH-5)
14	Almost 100% refractile spores; no granules (T); granular cytoplasm (CH-5)	Almost 100% refractile spores; homogenous cytoplasm (T); granular cytoplasm (CH-5)

Figure 1. Growth indicators of Bacillus cereus T in N- and PBG-media. Portions of culture (100 ml) from N-medium (O) and PBG-medium (●) were harvested at timed intervals and the pH (a), cell dry weight (b), and culture turbidity (c) were determined, as explained in Materials and Methods, and presented as a function of culture age.

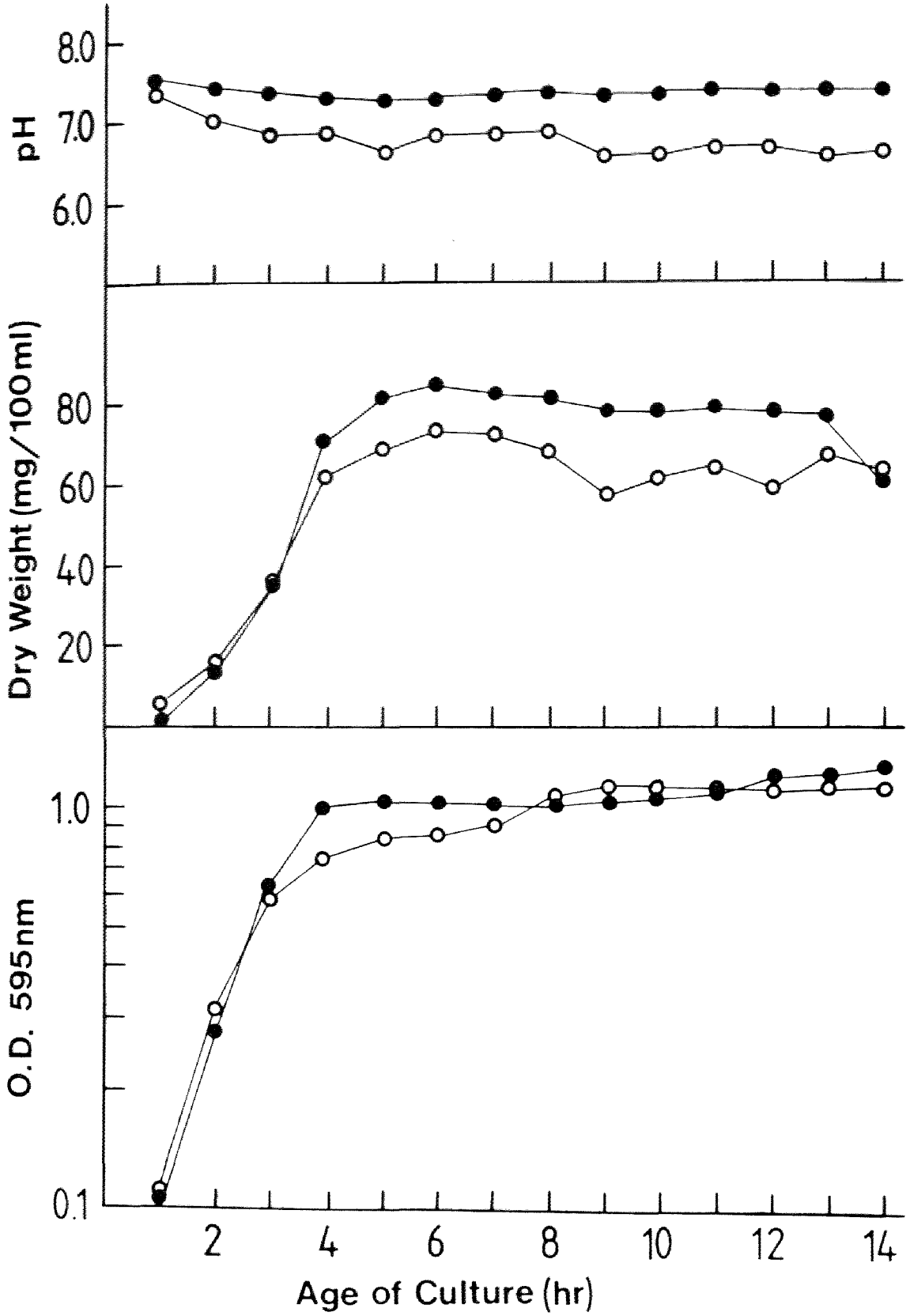
pH
8.0
7.0
6.0
Dry Weight (mg/100ml)
80
60
40
20
O.D. 595nm
1.0
0.1
2
4
6
8
10
12
14
Age of Culture (hr)

Figure 1

cell dry weight, and culture turbidity, respectively, of B.c.T as a function of age in N- and PBG-media. The pH profiles agree well with earlier studies (Slock, 1970; Berka, 1979; Schmid, 1980) and demonstrate the effectiveness of the buffer in the PBG-medium. Exponential growth stopped at approximately four hours in both media and later increases in turbidity were due to the development of refractile forespores, not to an increase in cell mass.

The growth parameters of medium pH, cell dry weight, and culture turbidity for CH-5 in N- and PBG-media are presented in Figures 2A, B, and C, respectively. These values agree very closely with those of B.c.T, indicating that the mutant has not suffered any major damage to the normal metabolic processes controlling growth and sporulation.

The pH of N-medium during growth of B.c.T dropped from an initial value of 7.3 to a value of 6.7 at five hours and remained at this approximate pH through the final nine hours of observation. This was, most likely, due to the presence of organic acids produced from the oxidation of glucose during this time period.

During the growth of CH-5 in N-medium, the pH dropped from 7.3 at zero hours to 6.7 at six hours and then rose to 7.0 by 14 hours. This could be explained by the continual use of intermediate acids, produced by the oxidation of glucose, for the production of polysaccharide by

Figure 2. Growth indicators of Bacillus cereus CH-5 in N- and PBG-media. Portions of culture (100 ml) from N-medium (Δ) and PBG-medium (▲) were harvested at timed intervals and the pH (a), cell dry weight (b) and culture turbidity (c), were determined, as explained in Materials and Methods, and presented as a function of culture age.

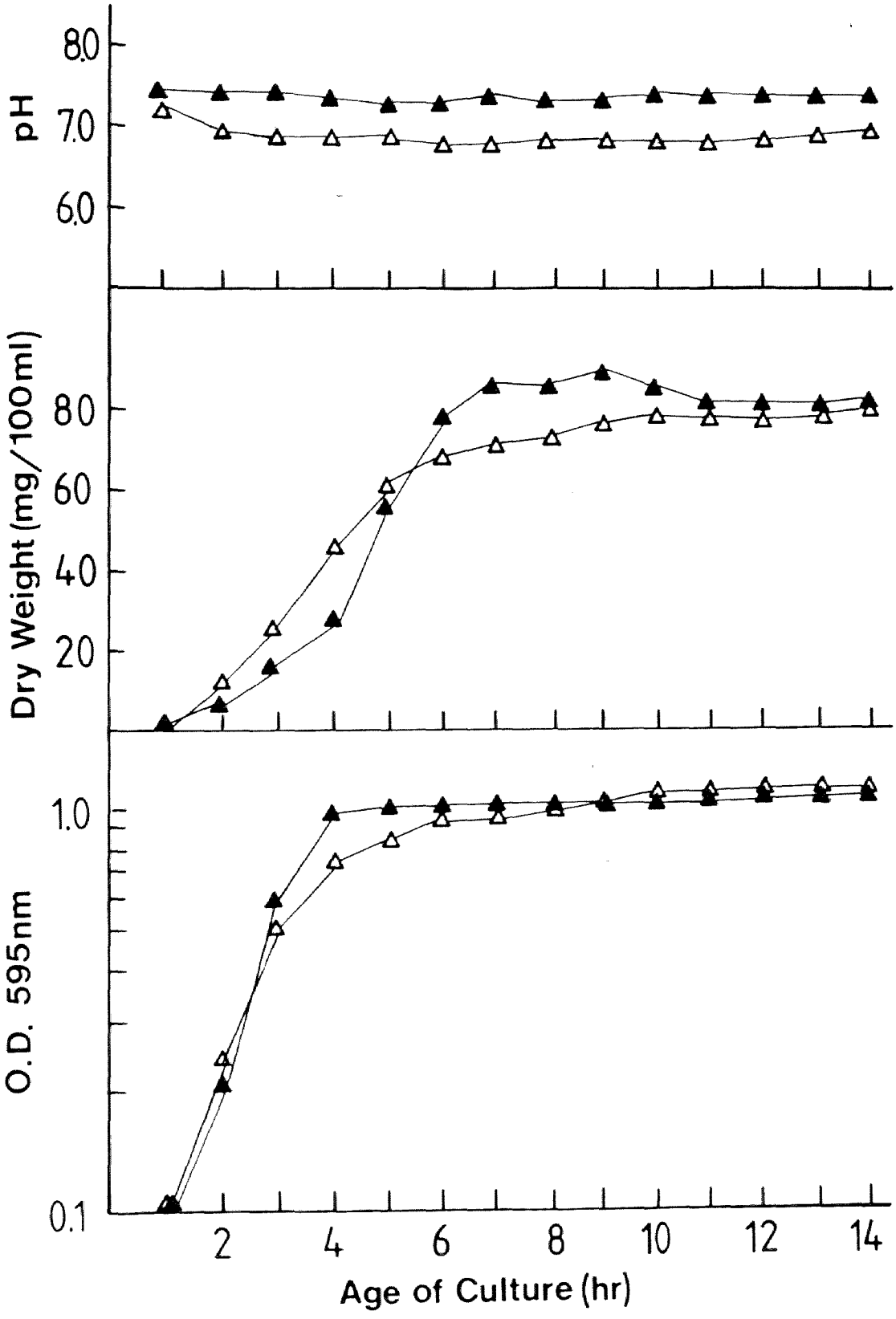
pH
8.0
7.0
6.0
Dry Weight (mg/100ml)
80
60
40
20
O.D. 595nm
1.0
0.1
2
4
6
8
10
12
14
Age of Culture (hr)

Figure 2

gluconeogenesis, and the inability of these cells to oxidize the polysaccharide to organic acids.

Total glucose content of whole cells. Slock (1970) demonstrated that most (if not all) glucose present in cells of B.c.T was in the form of polysaccharide. Therefore, the total glucose content of whole cells was used as an indication of the amount of intracellular polysaccharide present at various stages of development.

Cells from 100 ml portions of culture were harvested, hydrolysed in H_2SO_4, and assayed spectrophotometrically for glucose as explained in Materials and Methods.

The total glucose content of hydrolysates from B.c.T and CH-5 cells grown in N- and PBG-media are shown in Figures 3 and 4. In Figure 3, the values are given as mg glucose per 100 ml culture, and in Figure 4, as mg percent of dry weight.

The total glucose content was higher for both strains in N-medium compared to both strains in PBG-medium. This was due to the limiting supply of nitrogen and excess supply of glucose in the N-medium. Wilkinson (1959) showed that polysaccharide production was greatest in cells grown in a medium containing an excess of carbon and energy source and a limiting supply of some nutrient not essential for polysaccharide synthesis.

In N-medium, the total glucose content of B.c.T reached a peak of 15.9 mg (23 mg % dry wt.) at five hours

Figure 3. Total glucose content of whole cells (mg/100 ml) at different stages of development in N- and PBG-media. Portions of culture (100 ml) were harvested at timed intervals, hydrolysed with sulfuric acid, and the glucose content determined enzymatically as explained in Materials and Methods. Glucose concentrations of Bacillus cereus T in N-medium (O) and PBG-medium (●), and of CH-5 in N-media (Δ) and PBG-medium (▲) are expressed as a function of culture age. The culture turbidity (optical density at 595 nm) of N-medium (O) and PBG-medium (●) is included as an indicator of culture growth phase.

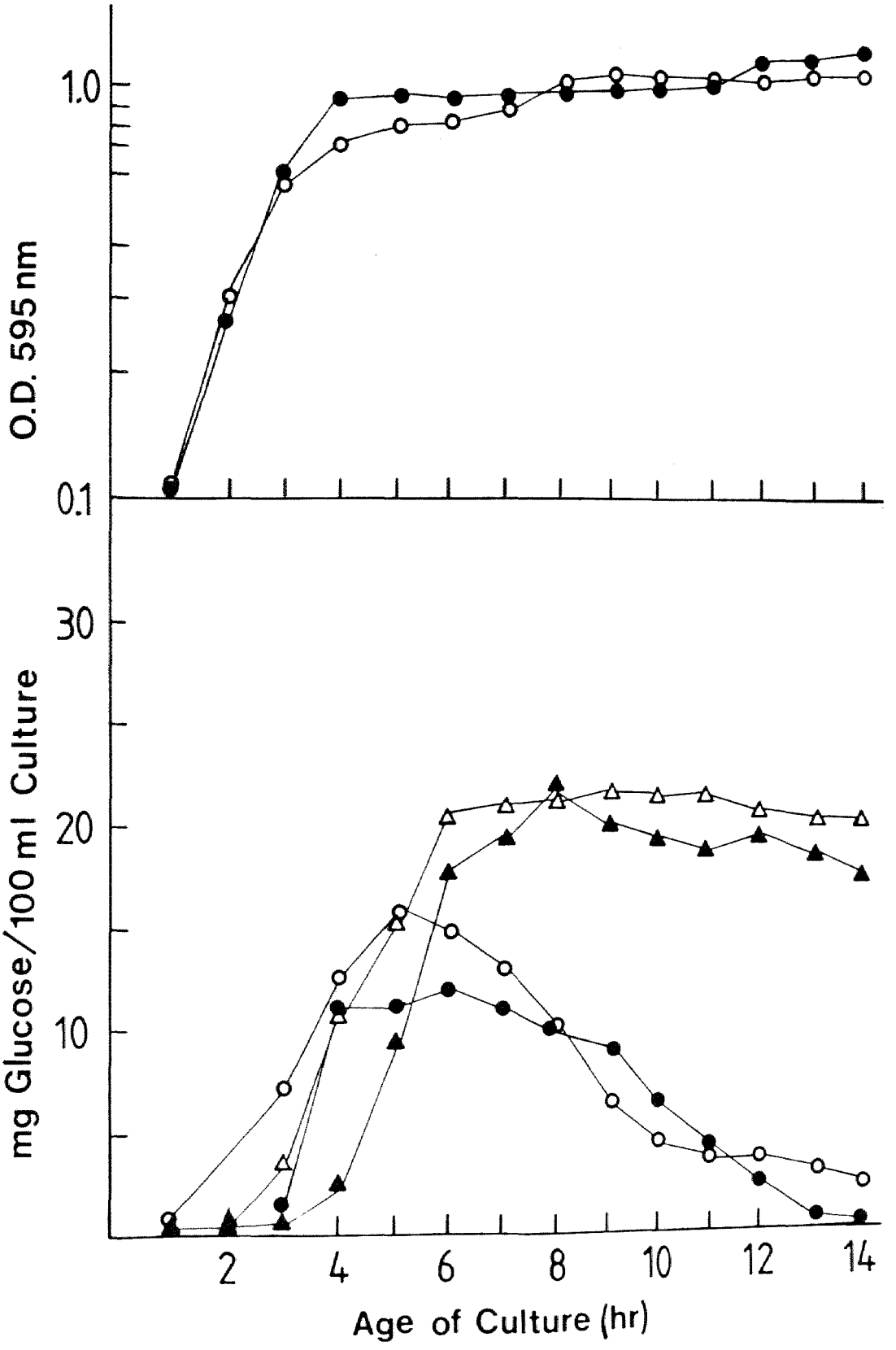
1.0
0.1
O.D. 595 nm
30
20
10
mg Glucose/100 ml Culture
2
4
6
8
10
12
14
Age of Culture (hr)

Figure 3

Figure 4. Total glucose content of whole cells (mg % dry weight) at different stages of development in N- and PBG-media. Portions of culture (100 ml) were harvested at timed intervals, hydrolysed with sulfuric acid, and the glucose content determined enzymatically as explained in Materials and Methods. Cell dry weights of the samples were determined as explained in Materials and Methods. The percent dry weight as glucose in Bacillus cereus T in N-medium (O) and PBG-medium (●), and in CH-5 in N-medium (Δ) and PBG-medium (▲) is expressed as a function of culture age. The culture turbidity (optical density at 595 nm) of N-medium (O) and PBG-medium (●) is included as an indicator of culture growth phase.

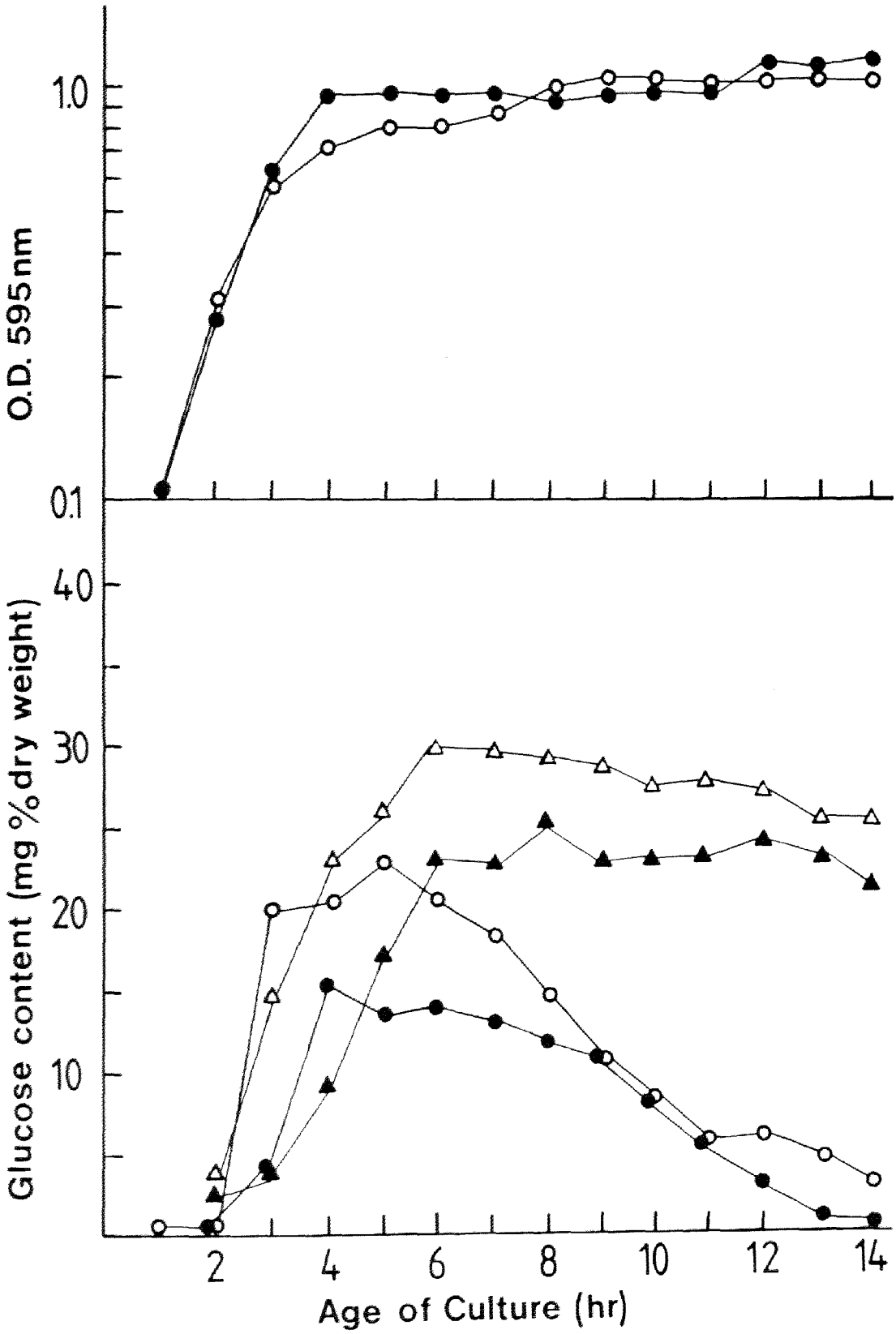
1.0
0.1
O.D. 595nm
40
30
20
10
Glucose content (mg % dry weight)
2
4
6
8
10
12
14
Age of Culture (hr)

Figure 4

and declined to 2.1 mg (3.3 mg %) by 14 hours. CH-5 glucose content reached a peak of 21.8 mg at nine hours (29.8 mg % at 6 hours) in N-medium. This level is essentially maintained through the development of refractile forespores, dropping to only 20.3 mg (25.5 mg %) by 14 hours.

The glucose content of B.c.T and CH-5 in PBG-medium reached respective peaks of 12.0 mg (14.1 mg %) at six hours and 21.9 mg (25.6 mg %) at eight hours. By 14 hours, B.c.T had fallen to 0.3 mg (0.5 mg %) glucose but CH-5 had maintained 17.6 mg (21.3 mg %) glucose per 100 ml culture.

The substantial drop in glucose content of B.c.T in both N- and PBG-media indicates that the polysaccharide was being degraded for use by the sporulating cells. It can be assumed that this process is beneficial to the cell and evidence presented earlier suggests that it may provide carbon and energy necessary for the formation of mature, heat-resistant spores (Slock, 1970; Berka, 1979; Schmid, 1980).

The polysaccharide content of CH-5 cells remained essentially constant throughout the stationary phase. This would indicate that carbon and energy for the completion of sporulation would have to come from some other source (exogenous glucose or intracellular PHB). Therefore, CH-5 cells should not be able to sporulate endotrophically after growth in PBG-medium which suppresses PHB accumulation.

The higher glucose content of CH-5 in N- and

PBG-media compared to that of B.c.T in the same media could be explained by the inability of CH-5 to degrade the polysaccharide. There may be a low level of polysaccharide degradation taking place throughout the growth curve of B.c.T which does not occur at all in CH-5.

PHB content of whole cells. It has been shown that PHB can serve as a carbon and energy storage compound for sporulation in B.c.T and that a major portion of PHB degradation products were used in spore synthesis, with 17% being incorporated into dipicolinic acid (DPA) and 50% into protein (Nakata, 1966). Poly-β-hydroxy butyrate (PHB) also accumulated to the greatest extent in cells grown in media containing an excess of carbon and energy source (Wilkinson, 1959). Kominek (1964) demonstrated that very little PHB accumulated in cells grown in media buffered at pH 7.4.

The PHB content of B.c.T and CH-5 cells at various stages of growth in N- and PBG-media was determined. Portions of culture (100 ml) were harvested, the PHB extracted, and the concentration determined spectrophotometrically as explained in Materials and Methods. The results are shown in Figures 5 and 6.

Cells of B.c.T and CH-5 grown in N-medium, accumulated maximum PHB contents of 6.5 mg/100 ml (11.3 mg %) and 6.6 mg/100 ml (9.0 mg %), respectively. These values declined to 3.6 mg/100 ml (5.7 mg %) and 3.7 mg/100 ml

Figure 5. Poly-β-hydroxybutyrate (PHB) content of whole cells (mg/100 ml) at different stages of development in N- and PBG-media. Portions of culture (100 ml) were harvested at timed intervals, autolysed in sodium hypochlorite, the PHB extracted, and the concentration determined spectrophotometrically as explained in Materials and Methods. The PHB contents of Bacillus cereus T in N-medium (O) and PBG-medium (●), and of CH-5 in N-medium (Δ) and PBG-medium (▲) are expressed as a function of culture age. The culture turbidity (optical density at 595 nm) of N-medium (O) and PBG-medium (●) is included as an indicator of culture growth phase.

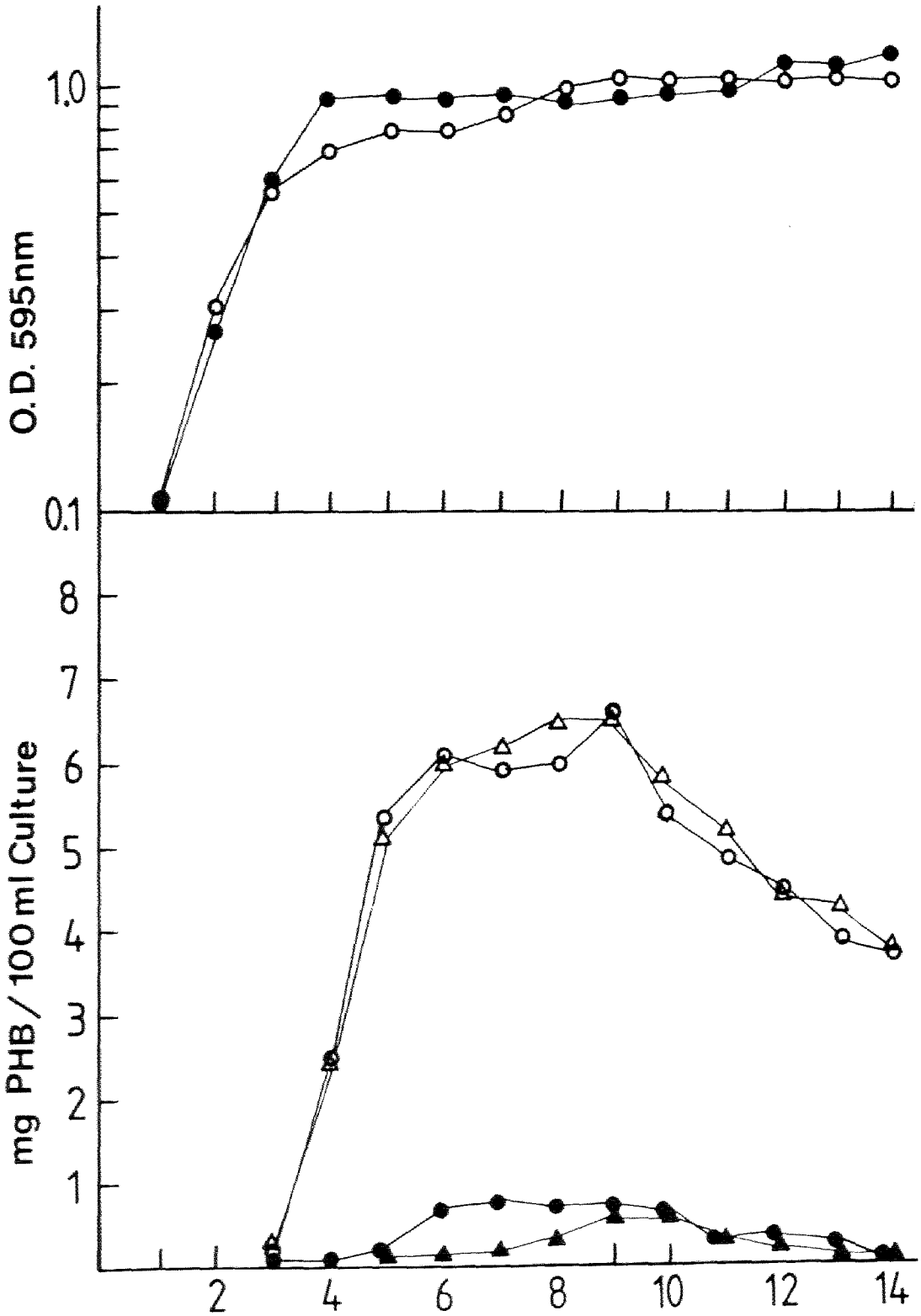
1.0
0.1
O.D. 595nm
8
7
6
5
4
3
2
1
mg PHB / 100 ml Culture
2
4
6
8
10
12
14
Age of Culture

Figure 5

Figure 6. Poly-β-hydroxy butyrate (PHB) content of whole cells (mg % dry weight) at different stages of development in N- and PBG-media. Portions of culture (100 ml) were harvested at timed intervals, autolysed in sodium hypochlorite, the PHB extracted, and the concentration determined spectrophotometrically as explained in Materials and Methods. Cell dry weights of the samples were determined as explained in Materials and Methods. The percent dry weight as PHB in Bacillus cereus T in N-medium (O) and PBG-medium (●), and in CH-5 in N-medium (Δ) and PBG-medium (▲) is expressed as a function of culture age. The culture turbidity (optical density at 595 nm) of N-medium (O) and PBG-medium (●) is included as an indicator of culture growth phase.

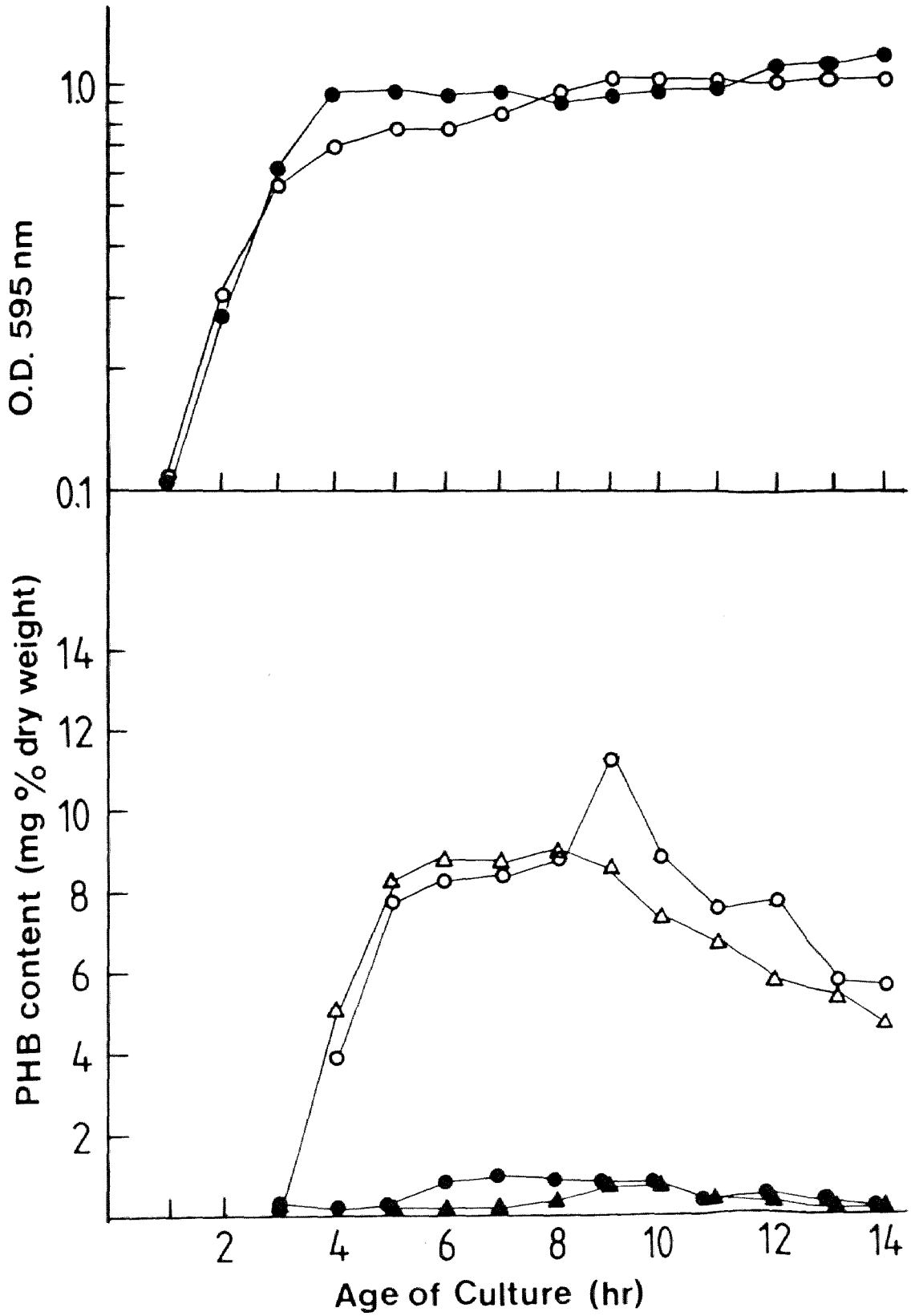
O.D. 595 nm
1.0
0.1
PHB content (mg % dry weight)
14
12
10
8
6
4
2
2
4
6
8
10
12
14
Age of Culture (hr)

Figure 6

(4.7 mg %), respectively, as sporulation proceeded.

In PBG-medium, B.c.T and CH-5 reached peaks of only 0.8 mg PHB/100 ml (1.0 mg %) and 0.6 mg PHB/100 ml (0.8 mg %), respectively. The PHB content then declined to 0.04 mg/100 ml (0.07 mg %) in B.c.T and 0.07 mg/100 ml (0.09 mg %) in CH-5.

The PHB content of B.c.T and CH-5 cells grown in PBG-medium is only about 10% of that in N-medium grown cells. This low level should not be enough to provide sufficient carbon and energy for the completion of sporulation. Under these conditions, the cells would require an alternative source of carbon and energy (exogenous glucose or intracellular polysaccharide) in order to complete the sporulation process.

The pattern of accumulation during early stationary phase and degradation during spore formation was consistent with previous studies (Wilkinson, 1959; Slepecky and Law, 1961a; Kominek, 1964; Nakata, 1966; Slock, 1970) and demonstrated that the mutant CH-5 is normal in this aspect of metabolism.

Ability of cells to sporulate endotrophically. Kominek (1964) demonstrated that PHB did not accumulate in cells grown in media buffered at pH 7.4. Under these conditions, a cell must have an alternative storage compound to provide the carbon and energy necessary for sporulation in a medium with no exogenous carbon and energy source. In

B.c.T, this alternative storage compound appears to be the intracellular polysaccharide.

Berka (1979) showed that a mutant of B.c.T, unable to accumulate the polysaccharide, was also unable to sporulate endotrophically after growth in PBG-medium. He also showed that a mutant producing an altered polysaccharide was able to sporulate endotrophically at only about half the efficiency of the wild-type.

The efficiency of endotrophic sporulation was determined as outlined in Materials and Methods. Briefly, cells were grown to early stationary phase (approximately 5 hours), washed twice, resuspended in a solution of 0.008% $CaCl_2.2H_2O$ in distilled water, and incubated. Counts were made before and after incubation.

Table 2 shows the efficiency of sporulation for B.c.T and CH-5 under various conditions. Both cell types sporulate at near 100% efficiency when allowed to sporulate in N- and PBG-medium. Bacillus cereus T also sporulates near 100% efficiency when grown in N- or PBG-medium and allowed to sporulate endotrophically. Bacillus cereus CH-5 cells grown in N-medium and allowed to sporulate endotrophically have an efficiency of sporulation comparable to that of the wild-type, but, when grown in PBG-medium and then placed under conditions of endotrophic sporulation, the efficiency of sporulation approaches 0%. This observation supports earlier claims that the intracellular polysaccharide

Table 2. Efficiency of sporulation of Bacillus cereus T and CH-5 under various conditions.

Cell Type	Growth Medium	Sporulation Medium	Efficiency of Sporulation
B.c.T	N	N	100%
		0.008% $CaCl_2.2H_2O$	96%
	PBG	PBG	100%
		0.008% $CaCl_2.2H_2O$	94%
CH-5	N	N	100%
		0.008% $CaCl_2.2H_2O$	91%
	PBG	PBG	70%
		0.008% $CaCl_2.2H_2O$	<10%

serves as a reserve source of carbon and energy for sporulation, especially under conditions preventing PHB accumulation (Slock, 1970; Berka, 1979; Schmid, 1980).

Heat-resistance of spores. The osmoregulatory cortex theory of spore heat resistance (Gould, 1978) maintains that heat resistance is brought about by osmotic dehydration of the forespore by the mother cell and is maintained by the osmotic activity of the peptidoglycan cortex and associated cations (primarily Ca^{++}). This suggests that heat resistance could be induced, or enhanced, by the presence of molecules in the medium, or mother cells, which raise the osmotic pressure.

Kawai and Tanaka (1952) demonstrated that high molecular weight compounds such as gelatin, starch, gum arabic, and alginic acid can exert a heat protective effect on spores of B. mesentericus and B. brevis. Stumbo (1965) established that high sugar concentrations increase the heat resistance of bacteria. Berka (1979) and Schmid (1980) found that B.c.T which produced an intracellular polysaccharide formed more heat resistant spores than polysaccharide-minus mutants of the strain. Berka (1979) postulated that the breakdown of the intracellular polysaccharide to glucose in the mother cell during sporulation could raise the osmotic pressure enough to aid in dehydration of the forespore and thus increase the thermostability.

Slock (1970) demonstrated that, approximately 0.3% glucose was still present in N- medium 10.5 hours after inoculation with an active culture of B.c.T. This glucose, present during sporulation, could act to increase the heat resistance of spores formed in N-medium. Slock (1970) also showed that glucose was exhausted from G-medium four hours after inoculation.

Heat resistance of B.c.T and CH-5 spores produced in N- and PBG-media, was determined by heating at 80°C for various lengths of time as described in Materials and Methods. The results are presented in Figure 7 and Table 3. The survivor curves (Figure 7) show the fraction of original cells surviving as a function of time at 80°C. The correlation coefficients represent the degree to which the points fit a straight line (1.00 is a perfect fit). The $D_{80°}$ values (Table 3) represent the length of time at 80°C required for the log survivor curve to transverse one log cycle and is equivalent to the inverse of the slope of the log survivor curve.

The survivor curve for B.c.T and CH-5 are consistent with previous findings (Schmid, 1980). The most heat resistant spores were produced by CH-5 in N-medium and the least resistant by CH-5 in PBG-medium. The correlation coefficients indicate relatively good straight-line trends for all four plots.

The $D_{80°}$ values for B.c.T spores in both media

Figure 7. Surviving fraction of spores as a function of time at 80°C. Spores were suspended in distilled water, heated for various lengths of time in an 80°C water bath, cooled, and the survivors enumerated by the pour-plate method as explained in Materials and Methods. The surviving fraction of spores, expressed as S_T/S_O (number of survivors at time T/number of survivors at time O), is expressed as a function of time at 80°C for B.c.T in N-medium (O) and PBG-medium (●) and CH-5 in N-medium (Δ) and PBG-medium (▲). The respective correlation coefficients represent the degree to which the points fit a straight line (1.00 being a perfect fit).

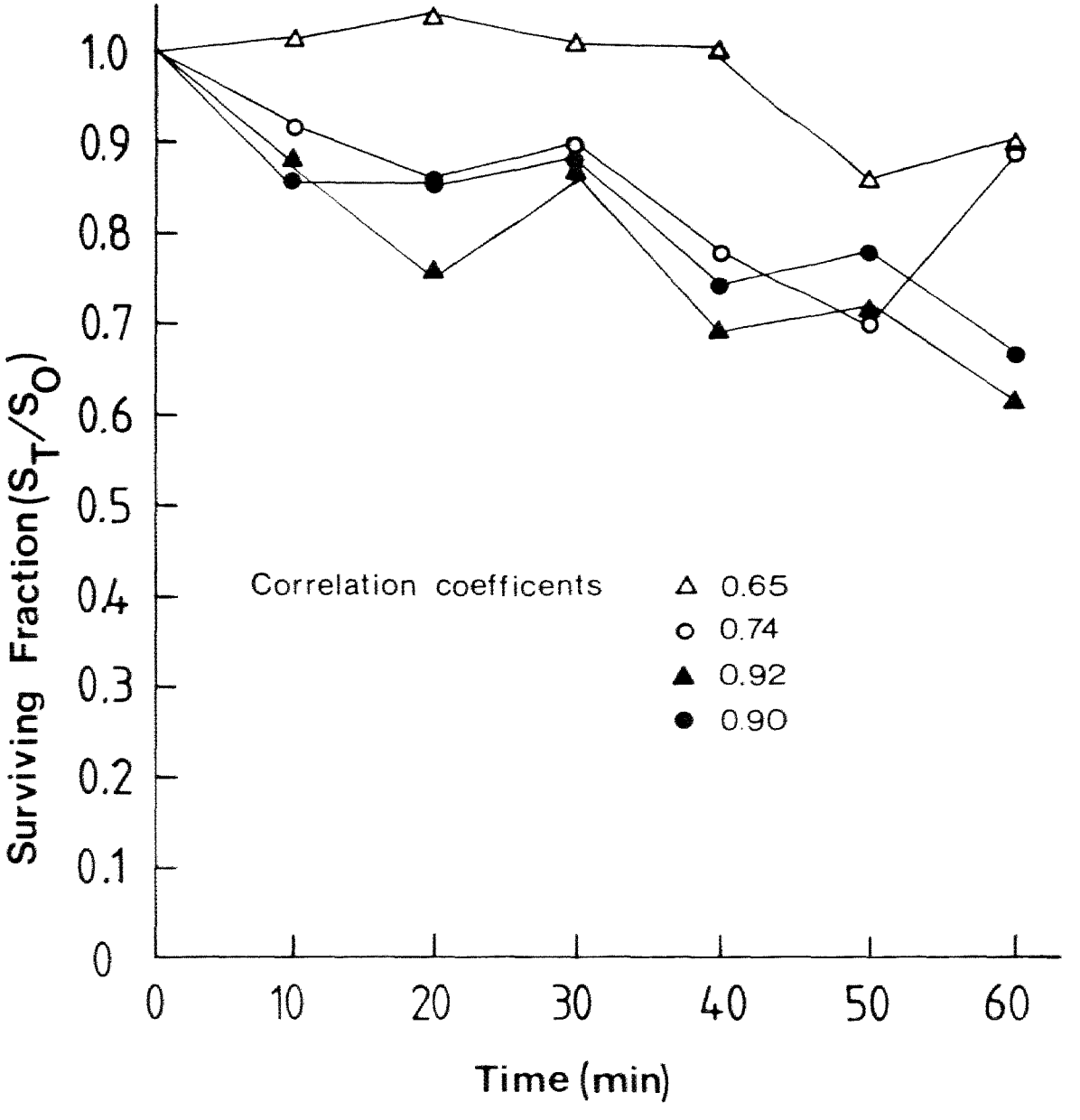
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Surviving Fraction (S_T/S_O)
0
10
20
30
40
50
60
Time (min)
Correlation coefficents
△ 0.65
○ 0.74
▲ 0.92
● 0.90

Figure 7

Table 3. Heat resistant of Bacillus cereus T and CH-5 spores produced in N- and PBG-media.

Cell Type	Growth and Sporulation Medium	$D_{80°}$ (min.)
B.c.T	N	661
	PBG	417
CH-5	N	951
	PBG	299

(Table 3) were very similar to those observed earlier (Berka, 1979). The small increase in heat resistance of spores from N-medium may be due to the presence of low levels of exogenous glucose not present in PBG-medium.

Spores of CH-5 produced in PBG-medium were very sensitive to heat compared to those produced in N-medium or B.c.T spores produced in N- or PBG-medium. This would indicate that polysaccharide catabolism is essential for the formation of mature, heat-resistant spores in the absence of intracellular PHB and an exogenous source of carbon and energy. The increased heat resistance of CH-5 spores produced in N-medium suggests that the polysaccharide could contribute (by increasing the osmotic pressure) to the heat resistance of spores, regardless of whether or not it was degraded by the mother cell, as long as there was an alternative source of carbon and energy for sporulation (exogenous glucose or intracellular PHB).

SUMMARY AND CONCLUSIONS

Bacillus cereus T accumulates an intracellular polysaccharide and poly-β-hydroxy butyrate during early stationary phase and degrades both compounds as the process of sporulation proceeds.

A mutant (CH-5) of Bacillus cereus strain T (B.c.T) was isolated which is apparently normal in all aspects of metabolism except for its inability to degrade the

intracellular polysaccharide storage compound. This mutant was compared to the wild-type, with regards to morphological characteristics, polysaccharide accumulation, PHB accumulation, efficiency of endotrophic sporulation, and heat resistance, in order to determine what role the intracellular polysaccharide plays in the ability of the cell to form mature, heat-resistant spores.

Two media used for growth and sporulation of B.c.T and CH-5 were N- and PBG-media. N-medium (Slock, 1970) contained an excess of glucose and a limiting concentration of nitrogen. Polysaccharide and PHB accumulation were enhanced in N-medium. PBG-medium (Kominek, 1964) was strongly buffered at pH 7.4 and growth was limited by glucose. Accumulation of PHB was depressed in PBG-medium.

No morphological differences were seen between B.c.T and CH-5 during growth in either medium. The cell yields, growth rates, and sporulation rates were virtually identical for the two strains under identical conditions.

The peak polysaccharide contents in N-medium were 15.9 mg/100 ml culture (23.0 mg % dry wt.) and 21.8 mg/100 ml culture (29.8 mg % dry wt.) for B.c.T and CH-5, respectively. In PBG-medium, the peak polysaccharide levels were 12.0 mg/100 ml (15.5 mg % dry wt.) and 21.9 mg/100 ml (25.6 mg % dry wt.) for B.c.T and CH-5, respectively. These peak values were reached during early stationary phase. In B.c.T., these values declined to 2.1 mg/100 ml (3.3 mg %)

and 0.3 mg/100 ml (0.5 mg %) in N- and PBG-medium, respectively, as refractile spores were formed. The peak levels of polysaccharide in CH-5 were essentially maintained throughout the sporulation phase, dropping to 20.3 mg/100 ml (25.5 mg %) in N-medium and 17.6 mg/100 ml (21.3 mg %) in PBG-medium.

The accumulation of PHB during growth in N-medium peaked at 6.5 mg/100 ml (11.3 mg %) in B.c.T and 6.6 mg/100 ml (9.0 mg %) in CH-5. In PBG-medium, B.c.T and CH-5 accumulated only 0.8 mg/100 ml (1.0 mg %) and 0.6 mg/100 ml (0.8 mg %) PHB, respectively. These values fell, as sporulation proceeded, to 3.6 mg/100 ml (5.7 mg %) and 3.7 mg/100 ml (4.7 mg %) for B.c.T and CH-5 in N-medium, and to 0.04 mg/100 ml (0.07 mg %) and 0.07 mg/100 ml (0.09 mg %) for B.c.T and CH-5 in PBG-medium. The relatively small amount of PHB accumulated and degraded during growth in PBG-medium is not enough to supply the cells with sufficient carbon and energy to complete the sporulation process.

The efficiencies of endotrophic sporulation for B.c.T grown in N- and PBG-media and for CH-5 grown in N-medium were all near 100%. But, CH-5 cells grown in PBG-medium had an efficiency of endotrophic sporulation near 0%.

Bacillus cereus T spores produced in N-medium were slightly more heat resistant than those produced in PBG-medium. The heat resistance of CH-5 spores produced in N-medium was higher than that of B.c.T spores produced in

N- or PBG-medium, and CH-5 spores produced in PBG-medium were substantially more sensitive to heat than spores produced by CH-5 in N-medium and B.c.T in N- or PBG-medium.

These results support the hypothesis that the intracellular polysaccharide does serve as a reserve source of carbon and energy for sporulation in Bacillus cereus T, especially under conditions preventing the accumulation of PHB. They also indicate that the presence of the polysaccharide may act to increase heat resistance of the mature spore even if it is not degraded for use as a carbon and energy source.

It would be interesting to examine mutants of Bacillus cereus T blocked at various stages of sporulation to see what effect the inability to sporulate has on the degradation of the polysaccharide and PHB.

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